

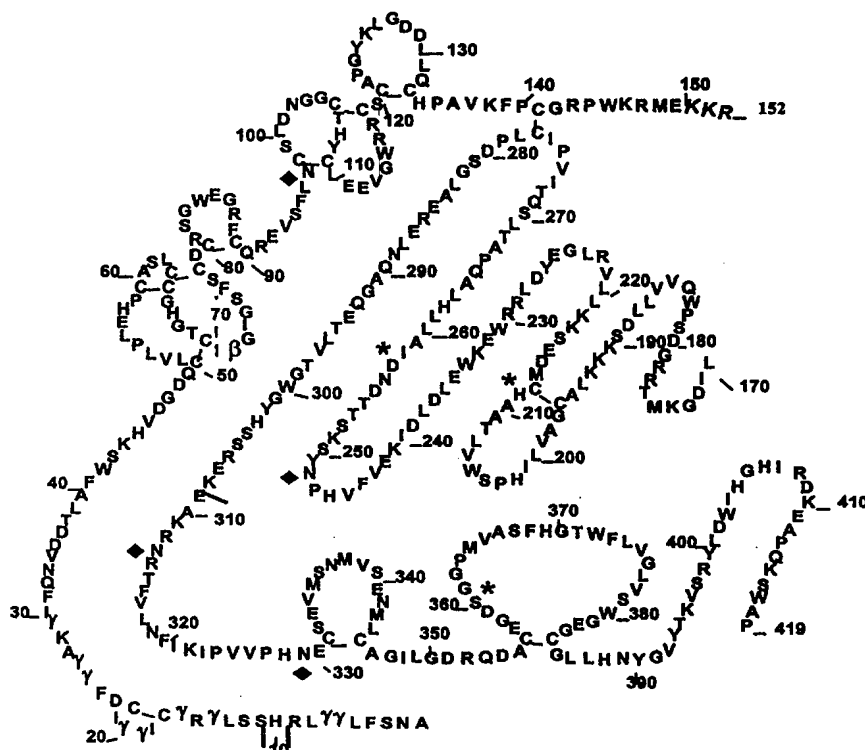


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(54) Title: IMPROVED METHODS FOR PROCESSING ACTIVATED PROTEIN C**(57) Abstract**

The present invention is broadly directed to a method for reducing autodegradation of activated protein C during processing and purification. The present invention provides aqueous activated protein C solutions and an improved method of processing of such solutions, comprising conducting such processing at an ionic strength of greater than 150 mM and at pH of about 5.5 to less than 6.3.



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Title

Improved Methods for Processing Activated Protein C

Priority

5 This application claims the benefit of U.S. Provisional
Application No. 60/045,255, filed 28 April 1997.

Field of the Invention

10 The present invention is broadly directed to a method
for reducing autodegradation of activated protein C during
processing and purification.

Background of the Invention

15 Protein C is a serine protease and naturally occurring
anticoagulant that plays a role in the regulation of
homeostasis by inactivating Factors V_a and VIII_a in the
coagulation cascade. Human protein C is made in vivo
primarily in the liver as a single polypeptide of 461 amino
acids. This precursor molecule undergoes multiple post-
20 translational modifications including 1) cleavage of a
42 amino acid signal sequence; 2) proteolytic removal from
the one chain zymogen of the lysine residue at position
156 and the arginine residue at position 157 to make the
2-chain form of the molecule, (i.e., a light chain of
25 155 amino acid residues attached through a disulfide bridge
to the serine protease-containing heavy chain of 262 amino
acid residues);

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3) vitamin K-dependent carboxylation of nine glutamic acid residues clustered in the first 42 amino acids of the light chain, resulting in 9 gamma-carboxyglutamic acid (GLA) residues; and 4) carbohydrate attachment at four sites (one in the light chain and three in the heavy chain). The heavy chain contains the well established serine protease triad of Asp 257, His 211 and Ser 360. Finally, the circulating 2-chain zymogen is activated in vivo by thrombin at a phospholipid surface in the presence of calcium ion.

Activation results from removal of a dodecapeptide at the N-terminus of the heavy chain, producing activated protein C (aPC) possessing enzymatic activity. In concert with other proteins, activated protein C functions as perhaps the most important down-regulator of blood coagulation resulting in thrombosis.

Unfortunately, aPC can autodegrade, leading to decreased functionality as an anticoagulant. An art recognized degradation pathway for activated protein C is a proteolytic clip at the lysine residue at position 308 of the heavy chain yielding a 111 amino acid fragment. This degradation product is recognized in the art as the EAK fragment.

Previous attempts to reduce autodegradation have focused on minimizing the formation of the EAK fragment. Most notably, Prouty et al., EP 0 662 513, July 12, 1995, teach minimizing autodegradation of aPC by controlling the pH to about 6.3 to 7.0; incubating the aPC in 3 M urea; or exposing aPC to extreme salt conditions, which are defined to be above 0.4 M or below 0.05 M.

Applicants have discovered a second important degradation pathway - autodegradation of the N-terminus of the light chain resulting in a clip on either side of the histidine residue at position 10. This degradation pathway yields two inactive products. The N-terminal clip of the first nine residues of the light chain yields des(1-9)activated protein C, and the N-terminal clip of the first ten residues of the light chain yields

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des(1-10)activated protein C. This degradation pathway, which has not been previously reported, results in loss of anticoagulant activity due to the removal of the critical GLA residues at positions 6 and 7. Therefore minimizing the level of the des(1-9)- and des(1-10)activated protein C autodegradation products is important in achieving a potent, high purity activated protein C pharmaceutical preparation. These variants were previously unknown degradation products and are exceedingly difficult, if not impossible, to remove by conventional purification techniques. The conditions to minimize their formation were previously unknown.

Identification of this important autodegradation pathway for activated protein C by Applicants has enabled the discovery of processing and formulation conditions to enhance the purity and potency of the activated protein C. Applicants have demonstrated that at a low pH (e.g. less than 6.3) the autodegradation pathway favoring the des(1-9)aPC or des(1-10)aPC predominates over the 308-309 autodegradation pathway, however use of elevated sodium chloride concentrations (greater than 150 mM) at a pH less than 6.3 substantially reduces the extent of the des(1-9)aPC and/or des(1-10)aPC autodegradation reaction.

Accordingly, the present invention provides for processing activated protein C at an ionic strength of greater than 150 mM and at a pH of about 5.5 to less than 6.3. Under these conditions the formation of des(1-9)aPC and des(1-10)aPC is significantly reduced. The present invention therefore provides an improved method for processing an aqueous solution of activated protein C without undesirable degradation.

Description of Drawings

Figure 1 provides the primary structure of activated human protein C to assist in illustrating the autodegradation pathways described herein. The nomenclature adopted herein is based on the numbering of the primary sequence of human activated protein C. One skilled in the

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art would recognize that other species of activated protein C may vary slightly in primary sequence thereby resulting in shifts in the nomenclature defined herein.

5 Summary of the Invention

The present invention provides aqueous activated protein C solutions and an improved method of processing such solutions, comprising conducting the processing at an ionic strength of greater than 150 mM and at a pH of about
10 5.5 to less than 6.3.

The invention further provides a method for purifying activated protein C by chromatographic separation, comprising eluting said activated protein C during said chromatographic separation using an aqueous elution solution
15 having an ionic strength above 150 mM and a pH of about 5.5 to less than 6.3.

The invention further provides a method for concentrating a solution of activated protein C by filtration, comprising feeding said activated protein C to a
20 filtration membrane as an aqueous solution having an ionic strength of greater than 150 mM and a pH of about 5.5 to less than 6.3.

The invention also provides activated protein C prepared by the processes described herein.

25 The invention finally provides activated protein C pharmaceutical preparations having less than 10% des(1-9)aPC and/or des(1-10)aPC by weight.

Detailed Description of the Invention

30 All amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. § 1.822(B)(2).

For purposes of the present invention, as disclosed and claimed herein, the following terms are as defined below.

35 aPC or activated protein C - refers to protein C whether recombinant or plasma derived. aPC includes and is preferably human protein C although aPC may also include

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other species or derivatives having protein C proteolytic, amidolytic, esterolytic, and biological (anticoagulant or pro-fibrinolytic) activities. Examples of protein C derivatives are described by Gerlitz, et al., U.S. patent No. 5,453,373, and Foster, et al., U.S. patent No. 5,516,650, the entire teachings of which are hereby incorporated by reference.

APTT - activated partial thromboplastin time.

Aqueous - includes cosolvent systems as well as use of water only as a solvent. Preferably, aqueous means water only as the solvent.

Chromatographic separation - includes chromatographic techniques recognized and appreciated in the art, including size exclusions, anionic, cationic, hydrophobic, reverse phase and the like.

Cross-flow filtration - refers to partitioning by tangential flow across a filtration membrane where the product is either retained by the membrane (as in concentration or diafiltration) or passed by the membrane (as in viral clearance filtration).

PC - protein C zymogen.

Processing - refers to unit operations useful in the manufacture of activated protein C, such as column chromatography, filtration (tangential, cross-flow, dead-end), lyophilization, pumping, or storage.

r-aPC - recombinant activated protein C produced by activating PC in vitro or by direct secretion of the activated form of protein C from procaryotic cells, eukaryotic cells, or transgenic animals including, for example, secretion from human kidney 293 cells as a zymogen then purified and activated by techniques well known to the skilled artisan and demonstrated in Yan, U.S. Patent No. 4,981,952, and Cottingham, WO 97/20043.

The present invention relates to an improved method for processing activated protein C. The invention particularly pertains to processing activated protein C through protein

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enrichment or concentration and protein purification operations, while reducing autodegradation. The invention is characterized by having such processing, and especially such modification, enrichment and purification operations, conducted using an aqueous solution of the protein under the condition described herein.

The combination of conditions claimed herein minimize the formation of des(1-9)aPC and des(1-10)aPC. Des(1-9)aPC and des(1-10)aPC are exceedingly difficult to remove by known purification methodology so it is desirable to minimize their formation during manufacturing of pharmaceutical preparations of aPC. Activated protein C prepared under the claimed conditions is substantially free of des(1-9)aPC and des(1-10)aPC variants. Generally, pharmaceutical preparations of aPC prepared under these conditions are substantively free of des(1-9)aPC and des(1-10)aPC, generally having less than 10%, preferably less than 8%, more preferably less than 5%, and most preferably less than 3% of these variants, individually or in combination, by weight. The lyophilized pharmaceutical formulations prepared using aPC prepared as described and claimed herein demonstrate improved stability in the solution state (prior to lyophilizing), and 24 to 48 hours stability upon reconstitution. Not more than a 5% loss in potency is observed up to five days at 2 to 8°C, up to 50 hours at 15°C, and up to 40 hours at 25°C. Prior to the present invention, such stability was previously unachievable.

Carefully controlling the pH, ionic strength, and preferably temperature, the autodegradation of activated protein C in aqueous solutions during processing and in formulations can be reduced to levels previously unobtainable - particularly in the absences of urea or other denaturing agents, histidine, lysine hydrochloride, or albumin.

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In the broad practice of the present invention, it is contemplated that processing protein C includes a wide variety of unit operations, physical separations and purification operations, including chromatographic treatment and cross-flow filtration such as for purifying the protein composition, concentrating the protein solution or for solvent exchange, as well as possibly chemical and enzymatic treatments. Protein processing contemplated by the present invention particularly includes purification by standard chromatographic methods such as ionic exchange chromatography, hydrophobic chromatography and the like, and protein concentration by ultrafiltration and similar processes. Protein processing is also intended to encompass the retention of the protein solution in holding tanks and the like preparatory to such purification and concentration steps. To reduce the instability, all processing of the activated protein C solution, including various protein purification and protein concentration steps, is conducted under the conditions described herein. Such processing steps are directed to the ultimate isolation of the protein, usually as a lyophilized powder, for formulation in a pharmaceutical preparation.

The pH of the aqueous processing solution is about 5.5 to less than 6.3. More preferably, 5.7 to less than 6.3. Still more preferably, a pH between about 5.6 to about 6.2. Even more preferred is a pH between about 5.8 to about 6.2. Still even more preferred is a pH between about 5.9 to about 6.1. The most preferred pH is about pH 6.0. Representative buffer systems to maintain effective pH control include Tris-acetate, sodium citrate, potassium citrate, citrate-glycine and sodium phosphate. More preferred buffer systems include sodium citrate and sodium phosphate. The most preferred buffer is sodium citrate. The preferred molarity of the buffer system is 10 mM to 50 mM. The most preferred molarity is 20 to 40 mM. The skilled artisan will recognize that many other buffer systems are available which also can be used to maintain the pH in the claimed range.

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The ionic strength of the processing solutions is the second critical element of the present invention. Ionic strength is generally derived from the addition of pharmaceutically acceptable salts - preferably sodium

5 chloride or potassium chloride. The ionic strength is preferably greater than or equal to 150 mM, derived from a salt concentration of greater than 150 mM in a buffered solution. Preferably, the salt concentration is no more than about 1000 mM to facilitate downstream processing.

10 Most preferably, the salt concentration is from about 200 mM to 1000 mM. Concentrations of greater than 50 mM and below 400 mM were previously believed to be unacceptable.

An additional condition to ensure minimal autodegradation is the temperature. Preferably, the

15 processing temperature during solution processing is between 0°C and 10°C, more preferably 2°C to 8°C outside of these temperatures, significant autodegradation of activated protein C occurs. However, exceeding 10°C for short periods of time can be tolerated without comprising the integrity of

20 the activated protein C.

The concentration of activated protein C is not critical to the present invention. Significantly, the ability to process the protein at high concentrations is significantly enhanced under the conditions described

25 herein. The preferred protein concentration is from about 1 mg/mL to about 50 mg/mL, more preferably 1 to 30 mg/mL, still more preferably 1 to 20 mg/mL, and most preferably 1 to 10 mg/mL, although higher or lower concentrations are considered operable.

30 Activated protein C prepared as described herein is useful in the treatment of a wide variety of acquired disease states involving intravascular coagulation, including thrombotic stroke, deep vein thrombosis, pulmonary embolism, peripheral arterial thrombosis, emboli originating

35 from the heart or peripheral arteries, acute myocardial infarction, disseminated intravascular coagulation, and

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acute pre-or postcapillary occlusions, including transplantations or retina thrombosis.

Activated protein C is ideally formulated in the lyophilized state with a bulking agent. The bulking agent is ideally selected so that it improves the solid-state stability of the molecule. Examples of such excipients are sucrose, trehalose, and raffinose. The skilled artisan will recognize that many other bulking agents are available which also can be used in activated protein C. The bulking agent concentration of the formulation is a critical formulation variable of the freeze drying process. A preferred bulking agent is sucrose at a concentration in the solution to be lyophilized of 15 to 30 mg/mL. The most preferred concentration of sucrose in the solution to be lyophilized is 15 mg/mL in a formulation of aPC at 2.5 mg/mL. The most preferred concentration of sucrose in the solution to be lyophilized is 30 mg/mL in a formulation of aPC at 5.0 mg/mL.

The following examples are presented to illustrate and explain the invention. The scope of the invention should not be considered as being limited to these examples. Unless otherwise indicated, all references to parts and percentages are based on weight and all temperatures are expressed in degrees Celsius.

Preparation 1

Preparation of Human Protein C

Recombinant human protein C (zymogen) was produced in Human Kidney 293 cells by techniques well known to the skilled artisan such as those set forth in Yan, U.S. Patent No. 4,981,952, the entire teaching of which is herein incorporated by reference. The gene encoding human protein C is disclosed and claimed in Bang et al., U.S. Patent No. 4,775,624, the entire teaching of which is incorporated herein by reference. The plasmid used to express human protein C in 293 cells was plasmid pLPC which is disclosed in Bang et al., U.S. Patent No. 4,992,373, the entire teaching of which is incorporated herein by reference. The

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construction of plasmid pLPC is also described in European Patent Publication No. 0 445 939, and in Grinnell et al., 1987, Bio/Technology 5:1189-1192, the teachings of which are also incorporated herein by reference. Briefly, the plasmid was transfected into 293 cells, then stable transformants were identified, subcultured and grown in serum-free media. After fermentation, cell-free medium was obtained by microfiltration.

The human protein C zymogen was separated from the culture fluid by an adaptation of the techniques of Yan, U.S. Patent No. 4,981,952, the entire teaching of which is herein incorporated by reference. The clarified medium was made 4 mM in EDTA before it was absorbed to an anion exchange resin (Fast-Flow Q, Pharmacia). After washing with 4 column volumes of 20 mM Tris, 200 mM NaCl, pH 7.4 and 2 column volumes of 20 mM Tris, 150 mM NaCl, pH 7.4, the bound recombinant human protein C zymogen was eluted with 20 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.4. The eluted protein was greater than 95% pure after elution as judged by SDS-polyacrylamide gel electrophoresis.

Further purification of the protein was accomplished by making the protein 3 M in NaCl followed by adsorption to a hydrophobic interaction resin (Toyopearl Phenyl 650M, Tosohaas) equilibrated in 20 mM Tris, 3 M NaCl, 10 mM CaCl₂, pH 7.4. After washing with 2 column volumes of equilibration buffer without CaCl₂, the recombinant human protein C zymogen was eluted with 20 mM Tris, pH 7.4. The eluted protein was prepared for activation by removal of residual calcium. The zymogen was passed over a metal affinity column (Chelex-100, Bio-Rad) to remove calcium and again bound to an anion exchanger (Fast Flow Q, Pharmacia). Both of these columns were arranged in series and equilibrated in 20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4. Following loading of the protein, the Chelex-100 column was washed with one column volume of the same buffer before disconnecting it from the series. The anion exchange column

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was washed with 3 column volumes of equilibration buffer before eluting the protein with 0.4 M NaCl, 20 mM Tris-acetate, pH 6.5. Protein concentrations of recombinant human protein C was measured by UV 280 nm extinction

5 $E^{0.1\%}_{1\text{cm}} = 1.81$.

Preparation 2

Activation of recombinant human Protein C

Bovine thrombin was coupled to Activated CH-Sepharose 4B (Pharmacia) in the presence of 50 mM HEPES, pH 7.5 at 4°C. The coupling reaction was done on resin already packed into a column using approximately 5000 units thrombin/mL resin. The thrombin solution was circulated through the column for approximately 3 hours before adding 2-aminoethanol (MEA) to a concentration of 0.6 mL/L of circulating solution. The MEA-containing solution was circulated for an additional 10-12 hours to assure complete blockage of the unreacted amines on the resin. Following blocking, the thrombin-coupled resin was washed with 10 column volumes of 1 M NaCl, 20 mM Tris, pH 6.5 to remove all non-specifically bound protein, and was used in activation reactions after equilibrating in activation buffer.

Purified rHPC was made 5 mM in EDTA (to chelate any residual calcium) and diluted to a concentration of 2 mg/mL with 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5. This material was passed through a thrombin column equilibrated at 37°C with 50 mM NaCl and either 20 mM Tris pH 7.4 or 20 mM Tris-acetate pH 6.5. The flow rate was adjusted to allow for approximately 20 min. of contact time between the rHPC and thrombin resin. The effluent was collected and immediately assayed for amidolytic activity. If the material did not have a specific activity (amidolytic) comparable to an established standard of aPC, it was recycled over the thrombin column to activate the rHPC to completion. This was followed by 1:1 dilution of the material with 20 mM buffer as above.

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The anticoagulant activity of activated protein C is determined by measuring the prolongation of the clotting time in the activated partial thromboplastin time (APTT) clotting assay. A standard curve is prepared in dilution buffer (1 mg/mL radioimmunoassay grade BSA, 20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN₃) ranging in protein C concentration from 125-1000 ng/mL, while samples are prepared at several dilutions in this concentration range. To each sample cuvette, 50 μ L of cold horse plasma and 50 μ L of reconstituted activated partial thromboplastin time reagent (APTT Reagent, Sigma) are added and incubated at 37°C for 5 min. After incubation, 50 μ L of the appropriate samples or standards are added to each cuvette. Dilution buffer is used in place of sample or standard to determine basal clotting time. The timer of the fibrometer (CoA Screener Hemostasis Analyzer, American Labor) is started immediately after the addition of 50 μ L 37°C 30 mM CaCl₂ to each sample or standard. Activated protein C concentration in samples are calculated from the linear regression equation of the standard curve. Clotting times are the average of a minimum of three replicates, including standard curve samples. Protein concentrations of recombinant activated protein C was measured by UV 280 nm extinction $E^{0.1\%}=1.85$.

25

Example 1

Chromatographic Separation of aPC

A solution of aPC having a conductivity of approximately 14 mMho and a pH of 5.7 was applied to a 9.5 cm x 9 cm (hxd) column of S-Sepharose Fast Flow cation exchange resin linked in tandem with a 25cm x 14 cm (hxd) column of Q Sepharose Fast Flow anion exchange resin. Both columns had been pre-equilibrated in 20 mM sodium citrate, pH 6.0, 150 mM NaCl. The column load was 10 grams of aPC per liter of anion exchange resin. The column was washed with >2 column volumes of equilibration buffer and step-

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eluted with 20 mM sodium citrate pH 6.0, 400 mM NaCl. All operations were performed at 2-8°C and at a linear flow rate of 60 cm/hr.

Although the aPC had a high activity (539 units/mg by APTT assay) and was quite concentrated during the chromatography (>20 g/L at peak) and in the aggregate mainstream (10 grams /L), the product remained stable. These observations were confirmed by reduced and non-reduced tryptic digest light chain mass spectrometry, which indicated no detectable (<2%) isoforms containing the 308-309 endoproteolytic clip nor the presence of des(1-9)aPC and des(1-10) (<5%).

Example 2

15 Virus Filtration

Activated protein C (4 mg/mL) in 20 mM Tris, 150 mM NaCl, and 20 mM EDTA buffer is filtered using a tangential flow filtration (Millipore Virusolve™ 180 membrane). Passage of aPC through the filter is facilitated by using a diafiltration buffer. The diafiltration buffer is 20 mM sodium citrate and 150 mM NaCl. A solution (10L), 4 mg/ml aPC in 20 mM Tris, 150 mM NaCl, and 20 mM EDTA buffer were filtered. The sodium citrate and sodium chloride buffer was used as a diafiltration buffer to yield a final volume of aPC solution of 21.5 L.

Example 3

Freeze Drying

A solution is prepared in a vial by adding the appropriate amount of sucrose, sodium chloride, and sodium citrate to a predetermined volume of r-aPC to yield 2.5 mg/mL aPC, 15 mg/mL sucrose, 325 mM sodium chloride and 20 mM sodium citrate at pH 6.0. The solution is lyophilized using a conventional freeze dryer to yield lyophilized aPC vials suitable for reconstitution and administration to a patient. The lyophilized formulations have less than 10% des(1-9)aPC and des(1-10)aPC.

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Example 4

Freeze Drying

A solution is prepared in a vial by adding the
5 appropriate amount of sucrose, sodium chloride, and sodium
citrate to a predetermined volume of r-aPC to yield 5 mg/mL
aPC, 30 mg/mL sucrose, 650 mM sodium chloride and 40 mM
sodium citrate at pH 6.0. The solution is lyophilized
using a conventional freeze dryer to yield lyophilized aPC
10 vials suitable for reconstitution and administration to a
patient. The lyophilized formulations have less than 10%
des(1-9)aPC and des(1-10)aPC.

The principles, preferred embodiments and modes of
15 operation of the present invention have been described in
the foregoing specification. The invention which is
intended to be protected herein, however, is not to be
construed as limited to the particular forms disclosed,
since they are to be regarded as illustrative rather than
20 restrictive. Variations and changes may be made by those
skilled in the art without departing from the spirit of the
invention.

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We Claim:

1. An improved method of processing an aqueous solution of activated protein C, comprising conducting such processing at an ionic strength of greater than 150 mM and a pH of about 5.5 to less than 6.3.
2. The method of Claim 1 wherein protein C is human activated protein C.
3. The method of Claim 2 wherein the pH is from 5.6 to 6.2.
4. The method of Claim 3 wherein such solution comprises sodium chloride.
5. The method of Claim 4 wherein the sodium chloride concentration is 200 mM to 1000 mM.
6. The method of Claim 5 wherein the processing is selected from the group chromatographic separation, freeze-drying, or filtration
7. The method of Claim 6 wherein the pH is buffered with sodium citrate.
8. A method of processing an aqueous solution of activated protein C, comprising conducting the processing in an aqueous solution having a sodium chloride concentration of 150 mM to 1000 mM and a pH about 5.8 to about 6.2 buffered with sodium citrate; and at a temperature of about 0°C to about 10°C.
9. The method of Claim 8 wherein the pH is buffered with sodium citrate.
10. A method of processing an aqueous solution of activated protein C, comprising conducting the processing in an aqueous solution having a sodium chloride concentration of 200 mM to 1000 mM and a pH about 5.8 to about 6.2; and at a temperature of about 0°C to about 10°C.
11. An aqueous solution of Claim 10, having less than 10% des(1-9)activated protein C and des(1-10)activated protein C by weight.

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12. An aqueous solution of Claim 11, having less than 5% des(1-9)activated protein C and des(1-10)activated protein C by weight.

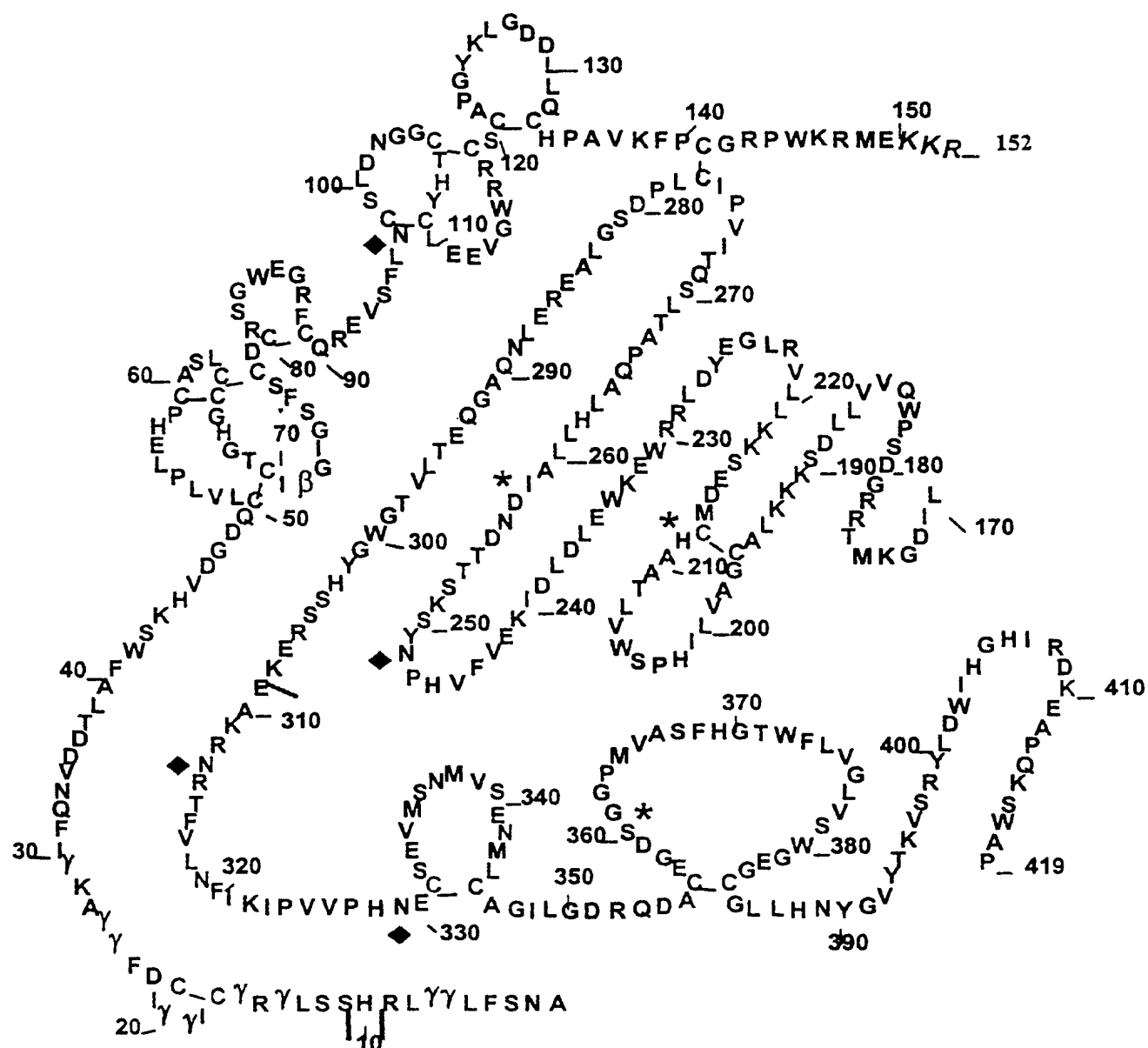
13. Activated protein C prepared from the process of
5 Claim 1.

14. A pharmaceutical formulation comprising activated protein C and a bulking agent; said formulation having less than about 10% des(1-9)activated protein C and des(1-10)activated protein C by weight.

10 15. A pharmaceutical formulation of Claim 14; said formulation having less than about 5% des(1-9)activated protein C and des(1-10)activated protein C by weight.

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FIG. 1



INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US98/08384
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61 K 38/00, 38/16, 38/17; CO7K 1/08, 14/435; C12P 21/02

US CL : 514/12, 21; 435/69.1, 69.6; 530/380, 381, 413

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21; 435/69.1, 69.6; 530/380, 381, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NAElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
NA**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,112,949 A (VUKOVICH) 12 May 1992 (12-05-92), see the entire document, especially column 8 (lines 40-50) and column 2 (lines 55 et seq).	1-10, 13 ----- 11-12, 14- 15
Y	US 4,849,403 A (STOCKER ET AL) 18 July 1989 (18-07-89), see entire document.	1-15

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

NATHAN M. NUTTER

Telephone No. (703) 308-0661